

Full Length Research Paper

Analysis of the *RNAi* targeting *FAD2* gene on oleic acid composition in transgenic plants of *Brassica napus*

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Oleate-¹² desaturase (*FAD2*) is a key enzyme involved in the conversion of oleic acid (C18:1) into linoleic acid (C18:2). *Brassica napus FAD2* gene was targeted for silencing by its *RNAi* gene under the control of a seed-specific napin promoter. This study aims to identify the transgenic plants, and analyze the level of *BnFAD2* transcripts in addition to fatty acid profile in *T₃* seeds. As indicated by PCR and southern blotting analysis, a total of six transgenic plants were developed. Analysis performed by RT-PCR revealed the significant down-regulation of *BnFAD2* transcripts in developing *T₃* seeds, which resulted in 13.90 to 32.20% increase of oleic acid composition in mature *T₃* seeds. The data demonstrated that *BnFAD2* gene was efficiently down-regulated and mediated by its *RNAi* gene, and oleic acid composition in transgenic rapeseeds was significantly enhanced.

Key words: *RNAi*, oleate-¹² desaturase (*FAD2*) gene, oleic acid, *Brassica napus*.

INTRODUCTION

Brassica napus L. is a major oilseed crop grown in many regions worldwide. Vegetable oils also represent a vast renewable resource of highly reduced carbon and those with a high content of oleic acid are of interest for nutritional and industrial purposes (Liu et al., 2002). The fatty acid composition of *Brassica* oil determines its physical and chemical properties. In general, the fatty acid of *Brassica* oil is composed of monounsaturated fatty acid (MUFA), fraction of oleic acid (C18:1) and polyunsaturated fatty acids (PUFA) namely: linoleic (C18:2) and linolenic acid (C18:3). High-oleic-acid content in the seed oil of *Brassica* is a current breeding objective because it increases the thermo stability of the oil, making it more suitable as cooking oil (Appelqvist et al., 1968). Further, oils with high oleic acid and low PUFA content are considered to be good for human consumption owing to the property of oleic acid to increase the level of high-density lipoproteins (HDLs) and reduce the level of low-density lipoproteins (LDLs) in blood (Chang et al., 1998). Therefore, increasing the

oleic and linoleic (O/L) ratio has become a major goal in this field (Broun et al., 1999).

Classical breeding programs can be designed to select increases in oleic acid content; however, this process requires a greater time investment than molecular manipulation. To overcome the limitations of conventional breeding approaches, genetic engineering techniques have been successfully employed to modify fatty acid composition in a number of oilseed crops (Kumar et al., 1991; Jadhav et al., 2005; Nabloussi et al. 2005). Many of the genes involved in the fatty acid biosynthetic pathway have been characterized and isolated. The enzyme¹² desaturase is known to be responsible for the conversion of the C18:1 into C18:2 which is further converted into C18:3 by the enzyme¹⁵ desaturase (Miquel et al., 1992; Browse et al., 1993). The microsomal 12 desaturase gene coding for the enzyme fatty acid desaturase (*FAD2*) is primarily responsible for more than 90% of the PUFA in non-photosynthetic tissues, such as roots and developing seeds of oilseed crops (Miquel et al., 1992). There have been a number of reports where the introduction of seed-specific antisense and sense *FAD2* constructs have led to a reduction in the PUFA levels in oilseed crops (Liu et al., 2002; Töpfer et al., 1995; Stoutjesdijk et al., 2000). In

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rapeseed (*B. napus*), transgenic lines have been developed with as high as 85% oleic acid and PUFA fraction reduced to 4 to 5% in their seed oils using co-suppression and antisense technologies (Kinney et al., 1994). A preliminary report by Stoutjesdijk et al. (1995), on increasing the oleic acid fraction in *Brassica juncea* (Australian variety) using co-suppression of *FAD2* reported an increase in the C18:1 fraction and a concomitant reduction in the C18:2 and C18:3 (PUFA) fractions.

RNA interference (*RNAi*) is a widespread silencing mechanism that acts at both the post-transcription and transcription. *RNAi* was first discovered by Guo and Kemphues (1995), when they noticed that the sense RNA inactivated the target gene as efficiently as did the antisense RNA. Fire et al. (1998) subsequently discovered that double stranded RNA (dsRNA) works much better than either sense or antisense RNA alone. RNA silencing occurs in a broad range of eukaryotic organisms, including fungi, animals and plants (Vander et al., 1990; Napoli et al., 1990; Romano et al., 1992). Gene silencing is an effective experimental tool to study gene functions in plants. At the same time, sequence-specific gene silencing has become more popular because of its powerful effect to inhibit the expression of a homologous endogenous gene. However, post-transcriptional silencing of plant genes using anti-sense or co-suppression constructs usually results in only a modest proportion of silenced individuals. Recent work has demonstrated the potential for constructs encoding self-complementary intron-hairpin RNA (ihpRNA) to efficiently silence genes.

In our previous study, we constructed the *RNAi* expression vector-pCAMBIA3301-fad2 targeting the gene *FAD2* according to *RNAi* strategy (Nabloussi et al., 2005). And then, using the cotyledons with petiole of rapeseed cultivar Y14 as explants, *RNAi* construct of the *FAD2* gene was introduced into rapeseed by *Agrobacterium tumefaciens*-mediated transformation (Stoutjesdijk et al., 2002).

In this report, we combined three methods of *GUS* histochemical staining, PCR screening, and southern blot to confirm the integration of *RNAi* into the transgenic plants. In addition, transcripts of *FAD2* gene and fatty acid profile were analyzed in T_3 seeds.

MATERIALS AND METHODS

Transformed with *RNAi* construct of the *FAD2* gene (*B. napus* L. vs Y14), a cultivar which was grown in the experimental field was obtained from Bioengineering Department of Zhengzhou University. Vector, pCAMBIA3301-fad2 and strains (*Escherichia coli* JM109) were kindly provided by Henan Key Laboratory of Crops Genetic Improvement. Restriction endonucleases DNA Marker, r-Taq were from TaKaRa Co. (Dalian, China); DIG DNA Labeling Kit were from Mylab CO. (Beijing, China); RNA Extraction Kit were from Watson Biotechnologies, Inc.(Shanghai, China). Tris-base, EDTA and CTAB were from GIBCO (Grand Island, USA). EB was from SIGMA Co. (St. Louis, USA).

GUS activity assay

The chromogenic substrate X-Gluc is used in a variety of applications for the detection of the -glucuronidase enzyme. Upon reduction, X-Gluc produces a localized colour, making it useful in identifying *GUS* gene presence in most cell types and for the detection of the *GUS* gene fusion marker in plants. *GUS* assay was carried out according to the method described by Jefferson et al. (1987) with some modification (Vissenberg et al., 2005). Segments of leaf from 1-week-old seedlings of T_2 generation plants were incubated in a solution containing 100 mM $\text{Na}_2\text{HPO}_4\text{-NaH}_2\text{PO}_4$ pH 7.0 buffer, 0.1% Triton X-100, 10 mM EDTA, 0.5 mM $\text{K}_3\text{Fe}(\text{CN})_6$, 0.5 mM $\text{K}_4\text{Fe}(\text{CN})_6$ and 1 mg/ml X-Gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid, cyclohexylammonium salt) at 37°C for 2 to 20 h. The stained tissues were then transformed into 95% ethanol for 24 h to remove chlorophylls. Wild-type rapeseed was used as control. Stained seedlings were observed under a Leica MZ APO binocular microscope (Leica, Cherboung, Switzerland), and images were recorded using a Canon PC1199 digital camera.

PCR analysis

DNA was isolated from leaf of T_0 transgenic plants by the CTAB method (Doyle et al., 1989). PCR reactions were carried out by r-Taq polymerase and buffer (TaKaRa Co.). The primer GUS-F: 5'-TCCCCGGGATCGAGGGCGCTTACGTCCTGTAGAAACCCC-3' and GUS-R: 5'-CGAGCTCTCATTGTTTGCCTCCCTGCTGCGGTT-3' were utilized to amplify a 1.8 Kb fragment from the genome DNA. The PCR amplification was run with the program: 5 min at 94°C, 1 min at 94°C, 1 min at 55°C and 2 min at 72°C for 35 cycles followed by 10 min at 72°C.

Southern blot analysis

Genomic DNA of transgenic and control plants was extracted and 15 g DNA was digested with EcoR I, after separation on a 1% agarose gel and transferred to a nylon membrane (Hybond N+, Amersham Pharmacia Biotech). A 572 bp Bar gene amplified from vector pCAMBIA3301 was labeled with DIG DNA Labeling Kit (Mylab Co.) for hybridization. After hybridization at 65°C for 26 h, the membrane was washed with 2x standard saline citrate (SSC), 0.1% SDS and 2.0 I Anti-Dig-AP for 15 min. The membrane was exposed to 200 I NBT/BCIP. The sequence of Bar primers is Bar-F:5'-CGAGTCTACCATGAGCCCAGAACGACGCC-3' and Bar-R:5'-GAAACTCGAGTCAAATCTCGGTGACGGGCAG-3'.

Semi-quantitative RT-PCR analysis

Developing seeds from T_1 and T_3 were harvested 30 to 35 days after pollination (DAP), frozen immediately in liquid nitrogen and stored at -70°C. Total RNA was isolated from seed using the RNA of Plant extraction kit (Watson Biotechnologies, Inc.) according to the manufacturer's instructions. DNase I (RNase-free) was used to digest the small amount of genomic DNA left in the extracted RNA samples, and then a control PCR using the extracted RNA as template was run to confirm the absence of genomic DNA in the RNA sample.

The RNA (1.5 µg) was reverse-transcribed (Primescript™ Reverse Transcriptase; TaKaRa Co.) according to the manufacturer's instructions and using an oligo-(dT)₁₅ primer. *B. napus -actin* gene was used as an internal control amplified by primers (BnA-F:5'-CGCCGCTTAACCCCTAAGGCTAACAG-3' and BnA-R: 5'-TTCTCTTTAATGTCCACGGACG ATTT-3'), which yielded an amplification product of 332 bp. The *FAD2* gene specific primers

(Fad2-F: 5'CGGAGAACTCAAGAAAGCAATC3' and Fad2-R 5'CAGACGAGTCA TAGTGAGGCAG3') yielded an amplification product of 761 bp. The amount of template cDNA and the number of PCR cycles were determined for each gene to ensure that amplification occurred in the linear range and allowed good quantification of the amplified products. The semi-quantitative RT-PCR amplification was run with the program: 5 min at 94°C, 1 min at 94°C, 1 min at 55°C and 1 min at 72°C for 23 cycles followed by 10 min at 72°C.

Fatty acid analysis

The oleic acid content was spectrophotometrically measured as described by Near infrared spectroscopy (FOSS NIRsystem 5000). NIR spectroscopy has been used successfully to differentiate canola seed with various level of oleic acid. NIR is an indirect analytical tool because it requires referencing to the analytical data acquired by means of *in vitro* wet chemistry techniques or *in vivo* derived data. It has been widely used for estimating the analyte content or the measurement of the functional property within the sample in a large number of samples of the same origin, eg. oil, protein and oleic acid content in rapeseed (FOSS NIRsystem). Self-pollinated seeds of *T₃* transgenic lines are the samples that enable recording of spectra from sample handling accessories.

Alterations in the activity of the 12-desaturase caused by the action of introduced transgene could be seen as changes in the amounts of oleic acid and in the seed oil profiles. An additional indirect method of assessing the cumulative effects of 12-desaturase activity during seed fatty acid synthesis is through the oleic desaturation proportion (ODP) parameter (Vander Krol et al., 1990), derived by the following formula: $ODP = [\% (18:2) + \% (18:3)] / [\% (18:1) + \% (18:2) + \% (18:3)]$. ODP represents the ratio of the total fatty acids accounting for the products of 18:1 desaturation (18:2 and 18:3) to the total amount of C18 substrate that was available. This parameter is useful in illustrating the effects of the *FAD2* gene-silencing transgenes on the level of endogenous 12-desaturase activity.

RESULTS AND DISCUSSION

Identification of *T₀* transgenic plants

Eighty-four putative *T₀* transgenic lines were grown in soil under containment. X-Gluc is a substrate for -glucuronidase (*GUS*) encoded by *gusA*, a widely used reporter gene. To make sure that the phosphinothricin-resistant seedlings are positive transgenic rapeseed, leaves of these seedlings were cut and stained with X-Gluc for detecting *GUS* activity. Only seedlings with positive staining signals were grown in the field for further study. All the *T₀* transgenic plants were analyzed, and thirty-five plants were *GUS* positive. *GUS* protein confirmed by X-Gluc shows that the construct is expressed in the developing endosperm, suggesting that the construction of *RNAi* construct is effective. In order to identify further transgenic *B. napus*, plants were verified by PCR analysis and a fragment of 1.8 kb was obtained after electrophoresis (Figure 1). Of the analyzed thirty-five transgenic plants, fourteen were PCR positive and twenty-one plants were PCR negative for the presence of the transgene. The results indicated that the *RNAi*



Figure 1. Identification of the transformed *B. napus* plants by PCR analysis. Lane 1: The negative control (untransgenic); Lane 2: The positive control (transgenic with pCAMBIA3301 vector); Lanes 3 to 8: The transgenic *B. napus* (transgenic with pCAMBIA3301-FAD2 vector).

construction of inverted repeat *FAD2* fragments had been putatively integrated into transgenic plants, which remained to be confirmed by Southern blot analysis.

Southern blot analysis

Southern blot analysis of genomic DNA was used to confirm the integration of the transgene into the *B. napus* genome and to estimate the transgene copy number with wild type rapeseed (*B. napus* L. vs. Y14) used as control. This result confirmed that portion of PCR positive plants contained Bar gene fragment, and also suggested that a portion of transgenic plants had single transgene copy (Figure 2), lane 6 negative control (untransgenic *B. napus*); lane 7, positive control (transformed pCAMBIA330 vector). Lanes 1, 2, 10 and 12 exhibiting a single band were recorded as single; lane 8, exhibits two bands. Six out of fourteen analyzed *RNAi* transgenic plants were found to have single copy insertions. Transgenic plants harbouring a single copy of transgene were selected for further analysis.

Expression level analysis

Semi-quantitative RT-PCR analysis was performed to determine whether the *RNAi* suppression in the transgenic *B. napus* plants is specific to the expression of the endogenous *FAD2* gene. The total RNA was extracted from immature seeds of the *RNAi*-mediated transgenic *B. napus* plants for the semi-quantitative RT-PCR analysis.

The expression level of the *FAD2* gene was monitored by semi-quantitative RT-PCR using endogenous gene primers. As shown in Figure 3, the expression level of *FAD2* gene in *B. napus T₁* transgenic lines decreased, but no change was found in the positive and negative controls. The *T₁* seeds from these plants were analyzed by semi-quantitative RT-PCR and fatty acid analysis, then the best six plants were grown to yield the *T₂* generation.

In the *T₃* seeds, semi-quantitative RT-PCR results (Figure 4) showed that the expression level of *FAD2* gene in four out of the six transgenic plants decreased significantly. Slight silence was brought about in the other two transgenic plants. These results proved that we had

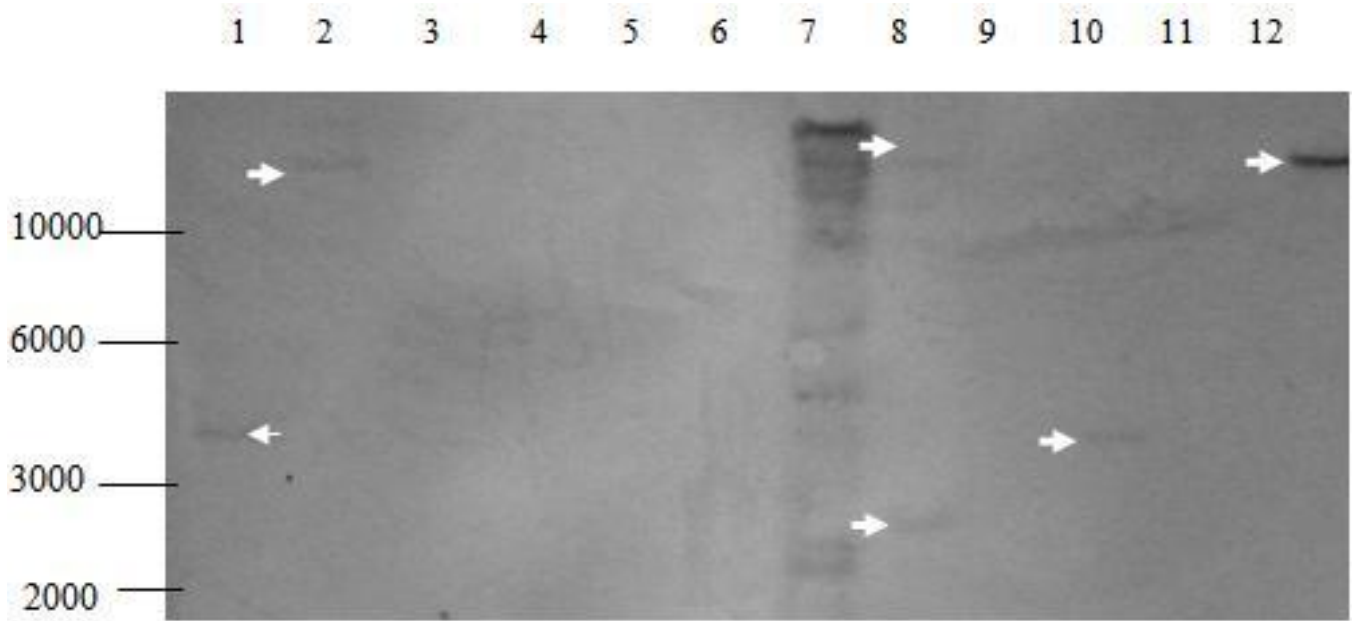


Figure 2. Southern blot analysis showing the number of integrations of the *RNAi* construct. Lane 6, negative control; Lane 7, positive control; Lanes 1 to 12 except 6 and 7, independent transgenic plants.

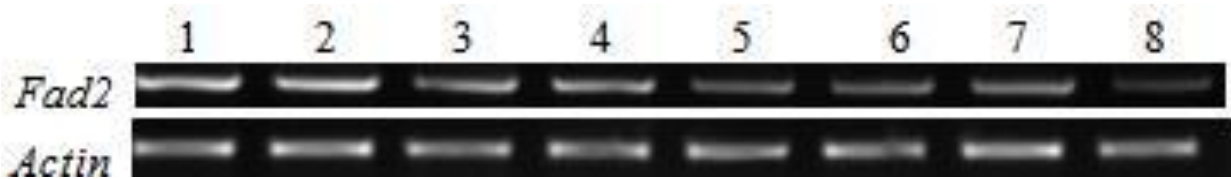


Figure 3. RT-PCR analyses on the mRNA levels of *FAD2* gene in *B. napus* T_1 transgenic lines. Lane 1: The negative control; Lane 2: The positive control; Lanes 3 to 8: The transgenic lines. Lanes 3, 4 and 7: slight silencing; Lanes 5, 6 and 8: obvious silencing.

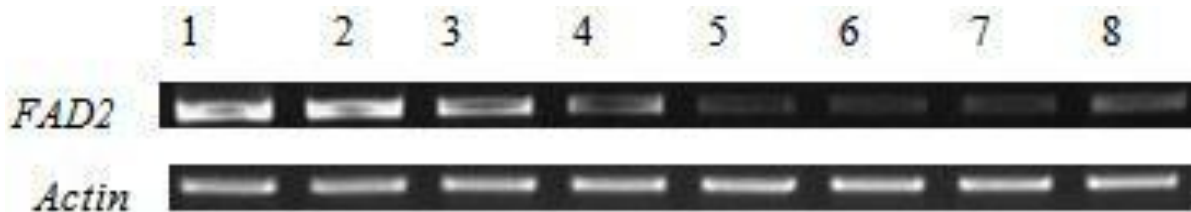


Figure 4. RT-PCR analysis on the mRNA levels of the *FAD2* gene in *B. napus* T_3 transgenic plants; Lane 1: The negative control; Lane 2: The positive control; Lanes 3 to 8: The transgenic plants; Lane 3: slight silencing; Lanes 4 to 8: obvious silencing.

acquired the *RNAi*-mediated transgenic plants that could produce silencing signal specific to *FAD2* genes in the seed.

Furthermore, this high degree of gene silencing achieved with *RNAi* construct was stably inherited between two generations. Primary transformant (T_1)

plants were hemizygous for transgene insertions. Therefore, the semi-quantitative RT-PCR analysis of the bulk seeds from self-pollinated T_1 plants would give an average value for each population of segregation genotypes, which would include some seeds that did not carry the transgene. But the section of T_3 seeds was

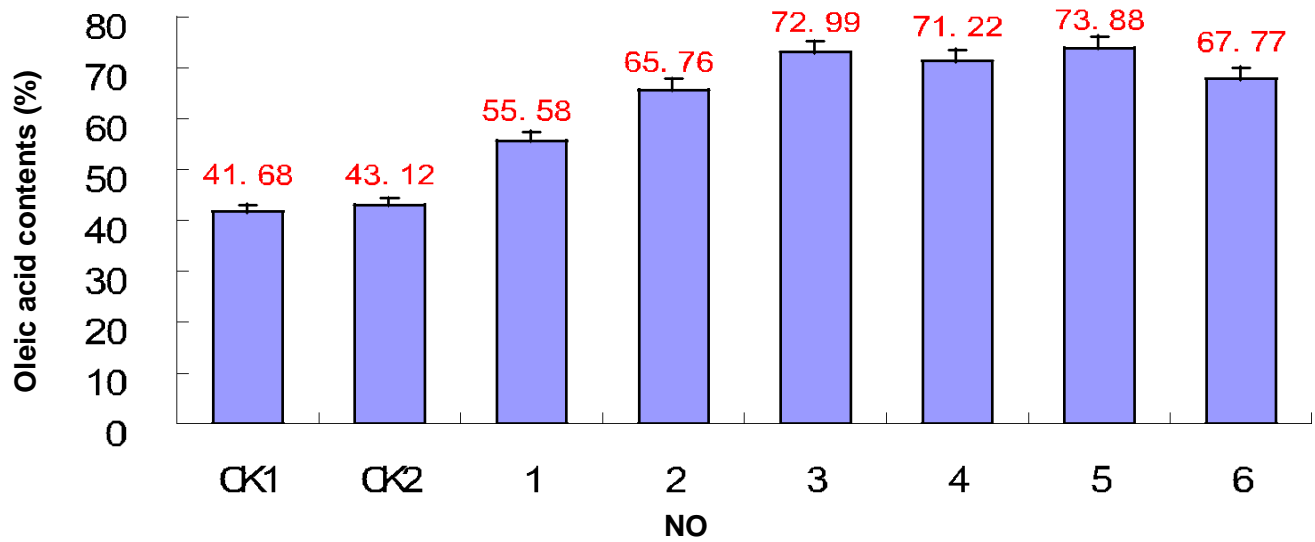


Figure 5. Oleic acid contents in total fatty acid of T_3 transgenic plants. WT: wide type; PC: positive control (transformed pCAMBIA330 vector); Bars1 to 6: transgenic plants.

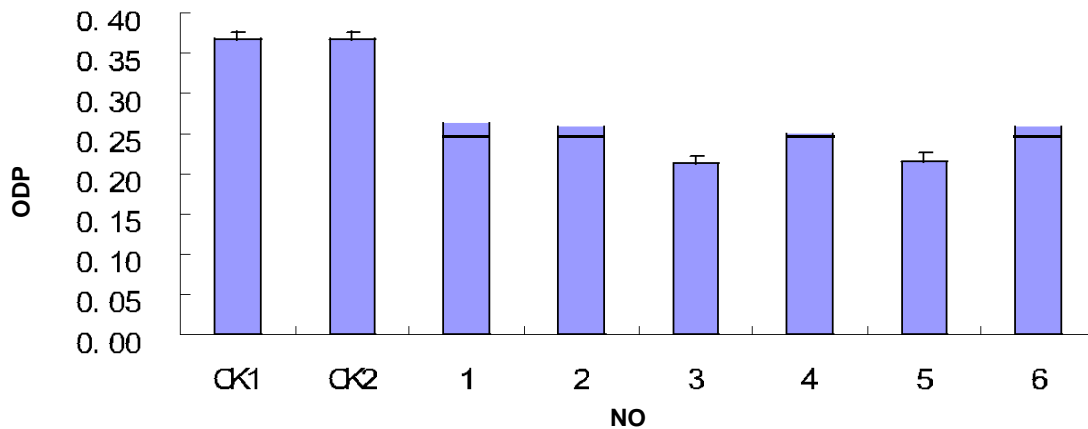


Figure 6. ODP value analysis in seeds of T_3 transgenic plants; WT: wide type; PC: positive control (transformed pCAMBIA330 vector); Bars1 to 6: transgenic plants.

homozygote, so the *FAD2* gene silencing levels of T_3 seeds was higher than T_1 seeds.

Analysis of oleic acid composition in T_3 seeds

The contents of the following fatty acids were determined: palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), linolenic acid (18:3), eicosenoic acid (20:1) and erucic acid (22:1). Fatty acids are expressed as % of the sum of all fatty acids. Seed oil content was determined by near-infrared-reflectance-spectroscopy (NIRS) expressed on seed dry matter basis.

The silence in *FAD2* gene activity led to a significant

increase in the oleic acid (C18:1) fraction in all the ten T_3 transgenic plants. All seeds from transgenic plants had oleic acid levels in the range of 55.58 to 73.88% (Figure 5). Transgenic plants showing the highest suppression in *FAD2* activity in the bar 3, 4 and 5, exhibited the highest mean oleic acid contents of 72.99, 71.22 and 73.88%, respectively, as compared to that of wide type (41.68%).

An ODP parameter can be used to assess the cumulative effect of *FAD2* activity. Herein, we used ODP to estimate *FAD2* activity in different transgenic line and wide type. In wide-type *B. napus*, the mean value was 0.366. Because oleic acid (C18:1) content increased significantly in transgenic plants, ODP value in these plants was much lower than that in wide type (Figure 6).

The fatty acid analysis from T_3 transgenic plants had considerable reductions in ODP value, and the ODP value decreased ranging from 0.103 to 0.154.

Data analysis showed that the *B. napus FAD2* gene has been selectively silenced by the *RNAi* construct. Both semi-quantitative RT-PCR and fatty acids analysis demonstrate that the greatly elevated oleic acid content in seeds of transgenic plant is due to the reduction in *FAD2* mRNA which resulted from PTGs. Furthermore, this high degree of gene silencing achieved with the *RNAi* construct was stably inherited in the transgenic lines. Stoutjesdijk et al. (2002) compared co-suppression, hairpin and intron-spliced hairpin constructs for reducing *FAD2* activity in *Arabidopsis*. The intron-spliced hairpin construct was shown to be the most effective, as this construct brought down the *FAD2* activity to as low as that reported in the null *fad2-1* mutant of *Arabidopsis* described by Okuley et al. (1994).

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